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14. ABSTRACT

Combinatorial library method significantly accelerates molecular discovery and identification in many areas of biology and medicine. Current applied array technique and split-mix approach both have their limitations. With this view, one-disc-one compound (ODOC) concept was first proposed and aimed to be applied to split-mix peptide library synthesis with the purpose of combining large-scale combinatorial synthesis and digital molecular identification as a whole. The constructed ODOC library may not only overcome the limitation of relatively small library size for array technique, but also greatly decrease the cost and tedious work of peptide sequencing for OBOC method through decoding the barcode on the discs. Therefore, the success of ODOC carriers for split-mix peptide synthesis may solve the bottlenecks of both array technique and OBOC method, increase the efficiency of drug discovery and even make great impact on modern drug industry.

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One-disc-one-compound carriers, barcode, split-mix, peptide synthesis

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Introduction

Combinatorial library method significantly accelerated molecular discovery and identification in many areas of biology and medicine, e.g., epitope mapping of antibodies, screening of cancer-targeting drugs and recognition of cellular receptors[1-3]. The up-to-date library synthesis strategy of combinatorial methods can be categorized as parallel array and split-mix approach[4-7]. Array technology can construct high density of molecules in an array format on a solid substrate (microchip), from which the chemical identity of each compound can be directly recognized by recording its location on the microarray. However, this approach greatly restricts the size of library and its inherent diversity. One-bead-one-compound (OBOC) method one of the most successful split-mix method, invented by Dr. Lam (Co-I) and his colleagues in 1991, has been widely applied for the peptide library synthesis due to its big library size (10^5-10^7) and great efficiency in drug discovery[8-9], however, it suffers from great cost of labor-intensive decoding procedure in order to obtain the sequences of synthesized peptides.

Prostate cancer is one of the most prevailing cancers and the second leading cause of death in Western countries. Up to now, chemotherapy is still the main treatment modality in prostate cancers[10-11], however the efficacy of the therapy is limited by severe toxic side effects induced by anticancer drugs on healthy tissues. Targeted chemotherapy which can be achieved by attaching a ligand for specific receptors that are expressed preferentially on malignant cells is intended to improve the efficacy of cytotoxic drugs against cancer cells, and meanwhile, reduce toxicity to normal tissues[12]. α6 integrin receptors are cell membrane receptors which have been found closely associated with the progression and metastasis of prostate cancer. Rosca et al. have developed a multivalent α6 integrin-specific construct with three identical peptide segments (-TWYKIAFQRNRK-), which can be used as the targeting probe for the directed delivery of drug or imaging agents[13]. Dr. Kit Lam (Co-I) reported a series of promising D-amino acid peptides with the minimal functional motif of (-kmvixw-), showing specific binding to α6 integrin as well as inhibiting invasion of prostate cancer cells[10, 14].

Microfluidics, in the past decade, has become a pervasive theme in the chemical reactions and biological analyses[15-16]. Benefiting from its miniature systematic dimensions (sub millimeter to micrometer), microfluidic system has significantly reduced the reagent volume and reaction time. Moreover, the inherent nature of microsystem enables the integration of different functional components in microfluidic system, which further improves the miniaturization and multiplexing of biochemical synthesis and analysis. However, up to now no example of microfluidic platform used for peptide library synthesis has been reported.

To address the above problems, one-disc-one compound (ODOC) concept was first proposed and aimed to be applied to split-mix peptide library synthesis with the purpose of combining large-scale combinatorial synthesis

and digital molecular identification as a whole. On one hand, Compared with array technique, the constructed ODOC library may overcome the limitation of relatively small library size, the library scale can probably reach the scale of OBOC (10^5-10^7); on the other hand, compared with OBOC method, with the encoded discs, the sequence of peptide on each disc can be easily obtained from decoding the barcode on the discs which greatly decrease the cost and tedious work of peptide sequencing. To achieve the concept of ODOC method, a microfluidic platform for peptide synthesis while keep tracking of the barcode history was established using a novel spinning technique. A 16-channel device was fabricated and tested to show that it can have a split capacity of 16 which will easily yield 16^4=65 536 individually different microdisc carriers in four spin motions (4 rounds of peptide synthesis with library size of 65 536). Utilizing the microfluidic split-mix synthesis and restricting chemical reactions onto microdisc surfaces, we may significantly facilitate the process of peptide synthesis and reduce amount of the reagent used. To further confirm the success of ODOC method, peptide library targeted to α6 integrin receptors will be constructed by ODOC method with the aim of discovery of new ligand for targeted chemotherapy and imaging of prostate cancer. Therefore, the success of ODOC carriers for split-mix peptide synthesis may solve the bottlenecks of both array technique and OBOC method, increase the efficiency of drug discovery and even make great impact on modern drug industry.

Keywords: ODOC carriers, split-mix peptide synthesis, targeted chemotherapy, α_6 integrin receptors

Overall Project Summary

1. Task 1.1 Fabrication of digitally encoded and biochemically activated microdisc carries.

Current objective:

To fabricate and release microdisc carriers using batch machining and to structurally embed recognizable barcodes into each individual microdisc carrier.

Results and discussion:

A polyethylene glycol (PEG) based photopolymerized composite was used as the structural and functional material for microdisc carrier due to its long term biocompatibility, optical clarity, capability to conjugate other functional derivatives, and most importantly, excellent swelling property in both polar and non-polar solvents, and also allowing high-purity peptide synthesis for solid-phase chemistry. To efficiently fabricate the microdisc carriers, a photo activated method was implemented and a polymer composite was obtained through optimizing different compositions. The optimized pre-polymerized mixture includes PEG-diacrylate (Mw 700, 16.4% w/w), 2-aminoethyl methacrylate hydrochloride (2.3% w/w), Dimethyl sulfoxide (DMSO) (48.1% w/w), photo-initiator (2-hydroxyl-2-methylpropiophenone, 2.3% w/w) and Benzyl acrylate (30.9% w/w). Under ultraviolet exposure, acrylate molecules can be polymerized with each other to form a highly insoluble matrix. The addition of 2-aminoethyl methacrylate hydrochloride forms an amine-terminated end group of matrix for

the subsequent peptide synthesis. In addition, 2.3% 2-aminoethyl methacrylate hydrochloride results in 0.23 mmol/g free amine loading in microdiscs, comparable to commercially available microbeads. To better decrease the non-specific bonding property of the carriers, we introduced benzyl acrylate into the mixture to modify the hydrophilic surface of the microdiscs. Different composition ratios of composite between structural material PEG and benzyl acrylate were prepared and compared through binding test on different surfaces (glass, PDMS and Polypropylene tube), and finally mass ratio of different compositions listed above were chosen for microdisc carriers fabrication.

The fabrication processes for microdisc carrier was shown in **Fig. 1**. As shown in **Fig. 1**, glass was used as a solid support and a spacer attached to the edge of glass surface defines the height of microdisc carrier. The direct contact of pre-polymerized solution with glass and photomask was performed, and after UV exposure for 20 s at a power of 15 mJ/cm^2, the microdisc carriers were formed, then released with buffer solution flow and collected in polypropylene tube. The microdisc carriers were then washed with alcohol to remove the unpolymerized PEG liquid. The obtained microdisc carriers can be stored in immersed buffer solution to get a monodisperse solution until use. 2-aminoethyl methacrylate hydrochloride can take part into the polymerization reaction which forms an amine-terminated end group of matrix for the subsequent peptide synthesis.

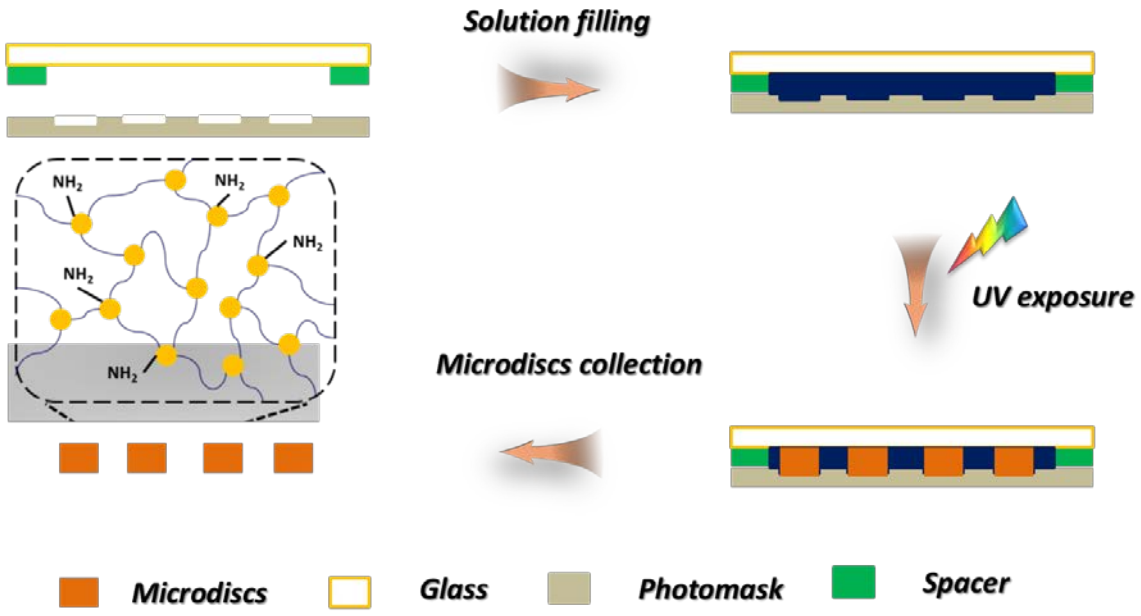

Figure 1. Fabrication process for microdisc carriers.

The lithographic resolution of photo-polymerized poly-ethylene glycol (PEG) based co-polymer was also defined (**Fig. 2a**). As shown in **Fig. 2a**, the microdiscs were 110 μm in diameter and 300 μm in center-to-

center distance. The size of each information bit is 10 μm and for orientation bit is 10-by-30 μm. Bars show 100 μm. We can easily control the geometry size of the microdisc carrier by fabrication parameters. First, the shape and diameter can be adjusted by the design of photomasks. For a carrier with diameter of 100 μm, 34×10^{19} ($=2^{35}$) digital sequences with a minimal lithographic resolution of 6 μm can be encoded. And the height can be controlled by using different spacers, which is selectively beneficial since this will influence the standing position of the microdisc carrier and furthermore influence the barcode readout process (**Fig. 2b-c)**. Therefore, through controlling the size of photomask and time of UV polymerization, the batch-fabricated array (10^6) of barcoded PEG microdisc carriers with amine-activated surface can be successfully achieved.

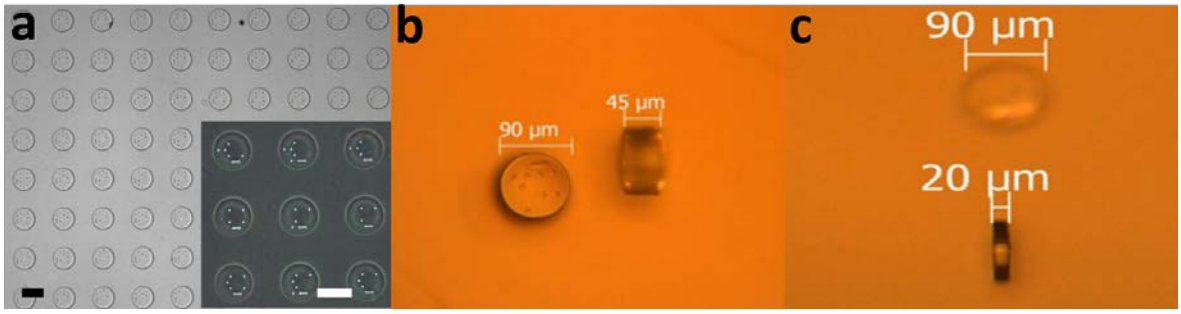

Figure 2. (a) Contrast image of fabricated digitally encoded microdisc array; (b-c) released microdiscs with different height.

Amine groups ($-NH_2$) are commonly used in chemical synthesis of peptide sequences on solid supports. **Fig. 3a** shows the Kaiser Test result of amine-functionalized microdiscs. As shown in **Fig. 3a**, the blue color clearly shows that the surface of PEG strip (same composition with microdiscs) was functionalized by free amine. A protecting-blocking-releasing partitioning method, previously reported by Lam's group, was applied to further modification of free amine. Specifically, the bulk-activated microdiscs will be first reacted with Fmoc-protected COOH derivatives, residual amine groups of PEG matrix will be irreversibly blocked by a mixture of acetic anhydride, N,N-diisopropylethylamine in DMF for 30 minutes. Subsequently, the Fmoc-protection group on the surface amines can be removed by immersing the discs in a piperdine solution of DMF to get the free amine again. **Fig. 3b** showed a group of released microdiscs with peptide synthesized by protecting-blocking-releasing partitioning method being screened by live cancer cells: some discs were covered by cells, meaning the peptides displayed on those discs had high affinity to the cells, while the others were not.

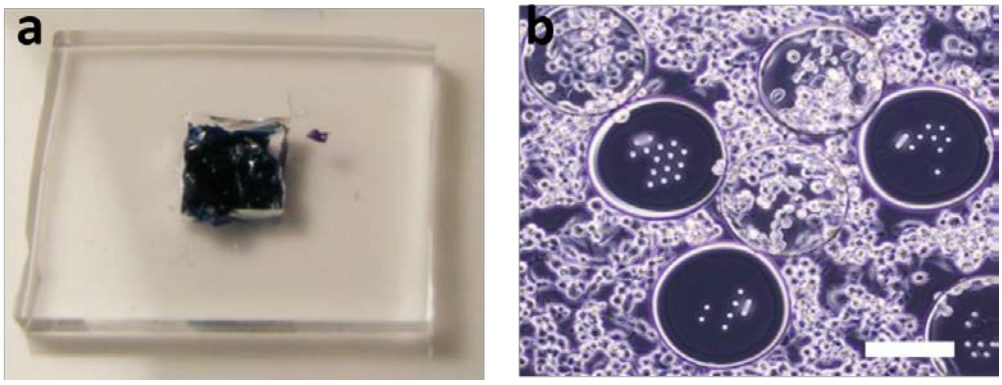

Figure 3. (a) Amine-functionized strip stained by Kaiser Test; (b) released microdiscs screened with live cancer cells.

To get a monodispersed solution of microdisc carriers, we dispersed them in a buffer solution composed of 25% (v/v) PEG-200 and 0.5% (v/v) Tween-20 in Phosphate buffered saline (PBS). Tween-20, a commonly used surfactant which can avoid strong interactions among microdisc carriers and decrease non-specific binding, make microdisc carriers monodispersed in the solution. The non-specific binding of the microdisc carrier was greatly influenced by contacting surface. The cylindrical shape of the microdisc carrier makes its bottom surface fully contact with substrate surface, which will result in large motion resistance. To solve this problem, a micropillar patterned PDMS surface was applied to decrease the motion resistance. The comparison of microdisc carrier adhesive state between patterned PDMS and unpatterned PDMS was shown in **Fig. 4**. As shown in **Fig. 4**, the majority of the microdisc carriers (blue dots) flow and attach to flat PDMS surface but not the patterned PDMS surface which means that the patterned PDMS surface can greatly decrease the non-specific binding of the microdisc carriers.

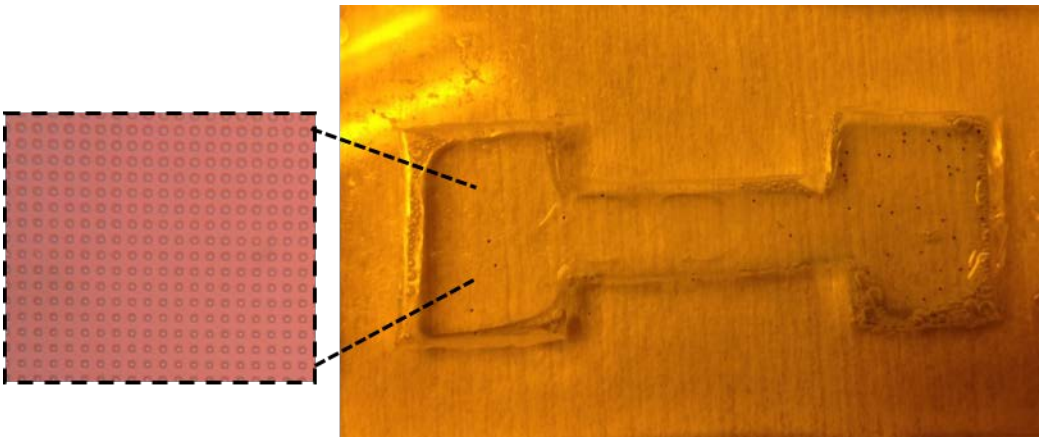

Figure 4. Adhesive behavior of microdisc carriers stained by bromophenol blue on micropillar patterned PDMS surface and flat surface. Left: patterned surface; Right: flat surface. Dimensions in patterned PDMS: 2 μm diameter of pillar, 6 μm center-to-center distance and 2.6 μm height of pillar.

In short, we achieved a highly efficient microdisc carrier fabrication and release protocol. Through controlling the size of photomask and times of UV polymerization, the batch-fabricated array (10^6) of barcoded PEG microdisc carriers with amine-activated surface were successfully achieved. A highly uniformly size distributed microdiscs with identical shape of cylinder were obtained, which forms a founded basis for peptide synthesis. The suspended microdisc carriers benefit from solution kinetics, ease of synthesis reactions, higher sample throughput and better quality control by batch synthesis[17]. Surface activation of free amine groups on PEG microdisc surfaces can be achieved by employing a protecting-blocking-releasing method. Besides, the non-specific binding of microdiscs is a major problem in disc distribution when doing peptide synthesis in microfluidic chambers, the micropillar surface of PDMS can greatly decrease the motion resistance and thus reduce the non-specific binding.

2. Task 1.2 Encoding and decoding strategies for the digital microdisc carriers.

Current objective:

To get a microscopic view of barcode readout and achieve a pattern recognizable microscopic picture sets.

Results and discussion:

A binary encoding scheme was used to design and recognize a distinctive digital-to-chemical identity on each microdisc (**Fig. 5**). As shown in **Fig. 5**, the design of encoding layouts on circular microdiscs consists of orientation (the bar indicating the front/backside of the disc) and information bits (the dots encoding unique digital sequences) parts. Without peptide sequencing, microscope scanning and recognition of barcode enable a highly efficient means to establish digital-to-chemical linkage between each numerical sequence and the synthetic identity on the corresponding disc. The barcode scanning system consists of a microscopic scanning set-up and pattern recognition algorithm. An Olympus IX81 inverted biological microscope equipped with PRIOR H117 motorized x-y stage can be employed to scan and image the barcoded discs during each synthetic step. In the next step, the scanned microdisc images can be processed and decoded using a pattern recognition algorithm programmed in Matlab (**Fig. 5**). Briefly, these microdisc images are first converted to grayscale pictures using object detection functions, from which the outlines and barcode patterns (information/orientation bits) can be recognized. Orientation of each microdisc can be then analyzed by locating its center and the orientation bit from the contrasted image. Subsequently, the information bits are then read and grouped to the corresponding microdisc. During each synthetic step, every microdisc will be imaged and recorded and the corresponding synthetic building block will be appended to its barcode sequence, which establishes an exclusive mapping from the numerical barcodes to the synthetic chemical sequences.

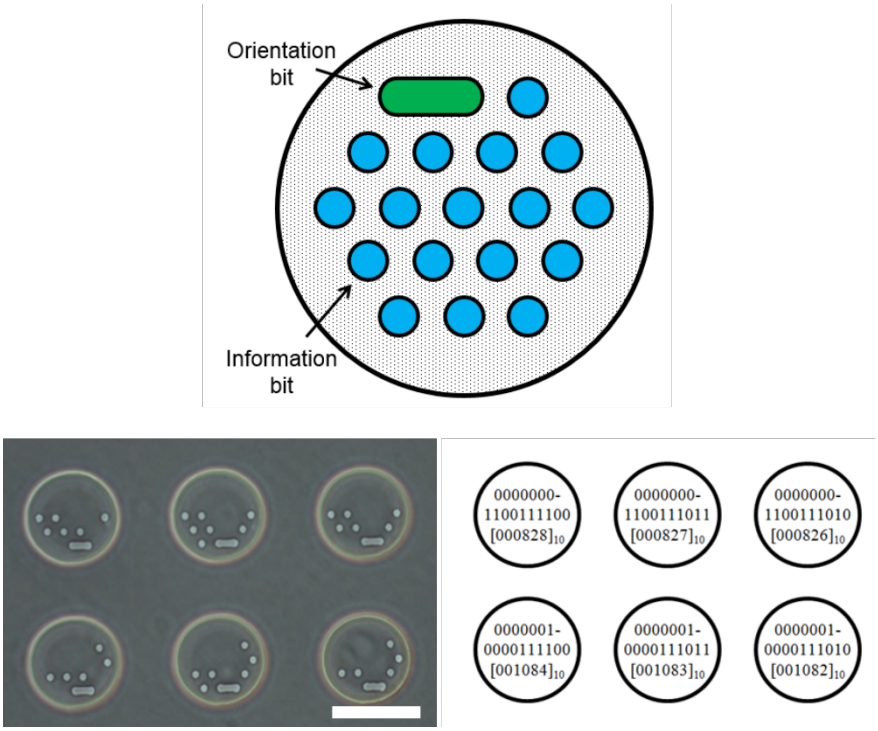

Figure 5. Barcode designs of microdiscs (top) along with contrast images (left bottom) and decoding numerical sequences of the microdisc array (scale bar: 200μm).

In short, a high-speed microscopic barcode-recognition system including both hardware and software are current being built. The design of software of a binary encoding strategy to provide each microdisc with an individually recognizable barcode in a large combinatorial library was constructed. However, the high-speed microscopic scanning setup which can be used to image individual microdiscs from massive microfluidic assembly in a monolayer configuration is still to be developed[18]. The precise microscopically captured images with distinguishable barcodes are rather important in linking the identified microdiscs to the synthetic combinatorial library by using the pattern recognition algorithm.

3. Task 1.3 Microfluidic synthesis platform and split-synthesize-scan-mix (S^3M) method

Current objectives:

A microfluidic synthetic platform will be established to adopt the highly efficient split-mix approach while allowing tracking the synthetic history of each element in parallel.

Results and discussion:

A PDMS based microfluidic device using centrifugal force as driving force was designed and fabricated (**Fig. 6)**. This device serves as a key component in the S^3M platform to undertake splitting and mixing microdisc carriers through centrifugal and pressurized fluidic force. As shown in **Fig. 6a**, The device has one inlet located in center and 16 outlet located at the far end of the plate, among which inlet will allow microdisc

carriers go into the spin channels and the outlet can let the flushing liquid out while keeping microdisc carriers stay in the channels. To retrieve microdisc carriers, positive pressure buffer solution was applied to drive them back into the center reservoir. In this way, we achieve a novel spin device which utilizes centrifugal and positive pressure force to drive microdisc carriers flow in a microfluidic channels. During the library synthesis, 16-channel spinning device, can split microdisc carriers by 16 parts individually under one spinning motion, then each building block can be synthesized in each microfluidic chamber. After block and Fmoc deprotection, the outer pressure force can then force the microdiscs back into the inlet and mix, and then split and mix again until the final building block reaction was finished. Therefore, this device provides a new technology for digital chemistry synthesis and recording by incorporating microscopic scanning in each round of peptide synthesis.

To collect the microdisc carriers at the outlet, we designed the chamber with a height enabled valve of 40 μm which is just smaller than the height of microdisc carriers. As shown in **Fig. 6b**, the microdisc carrier height (h_3, 50 μm), is slightly bigger than the height of spacing (h_2, 40 μm) between two sheet, and a stop sheet which has a height of 50 μm (h_1) can make the microdisc carrier stop at the outlet. Therefore, the channel for the microdisc carrier to flow through is within the height of h_1+h_2=90μ m.

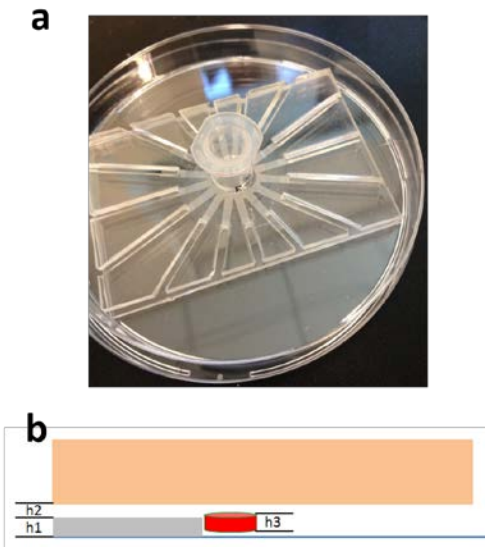

Figure 6. (a) 16-channel spin device for microdisc carrier splitting and mixing; (b) illustration of outlet of the spinning device (Dimensions: h_1=50 μm, h_2=40 μm and h_3=50 μm).

To achieve the functionalization of the device, the batch of microdisc carriers were prepared using the developed photopolymerized method and dispersed in buffer solution. Then the microdisc carriers were added into the inlet of the device, spin experiment was performed and microdisc carriers were collected in 16

channels as listed below **(Fig. 7**). As shown in **Fig. 7**, microdisc carriers are collected at the outlet, however, unevenly distributed which is not beneficial to the following peptide synthesis. However, it is believed that this could be avoided just by carefully modification of the device design and fabrication process.

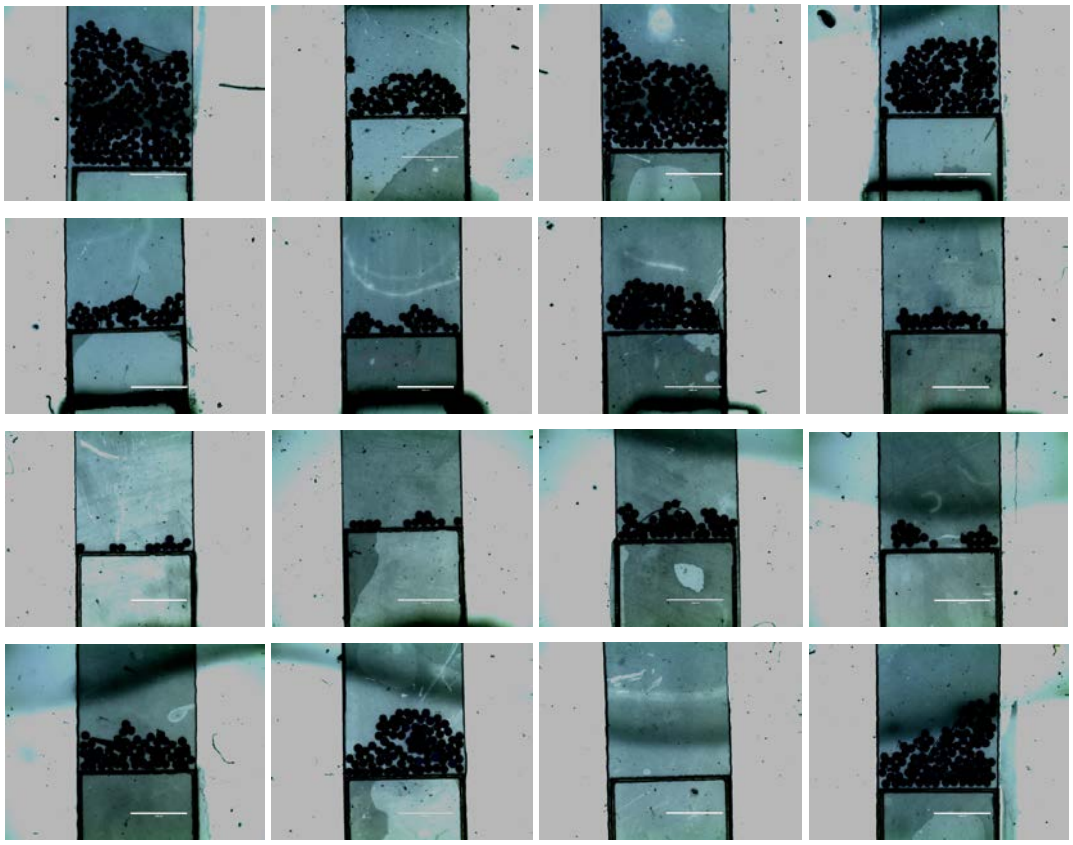

Figure 7. Microdisc carriers collected at the outlet of 16 channels.

In short, a microfluidic platform for peptide synthesis while keep tracking of the barcode history was established using a novel spinning technique. A 16-channel device was fabricated and tested to show that it can have a split capacity of 16 and can easily yield 16^4=65 536 individually different microdisc carriers in four spin motions (4 rounds of peptide synthesis with library size of 65 536). Although the collected microdiscs were unevenly distributed in 16 channels, it is believed that this could be avoided just by carefully modification of the device design and fabrication process.

4. Task 1.4 Preliminary result in design and synthesis of focused peptide libraries

Recently, the α4β1 integrin receptor has received much clinical interest because of its important role in metastasis and development of lymphocytic leukemia, bone cancer and myeloma. The important function and altered expression level of α4β1 integrin in cancer has made it an attractive target for cancer diagnosis and therapy. Therefore, there is a critical need to identify biomolecules targeting α4β1 integrin with high binding

affinity and specificity. To further confirm the accessibility of ODOC for split-mix synthesis, we tried the peptide library synthesis on glass, because of the same synthesis method used (protecting-blocking-releasing partitioning method), same encoding and decoding algorithm and some similar aspects in microfluidic synthesis, the success of peptide library array on glass by using microfluidic chip may make a strong foundation for the success of ODOC applied in split-mix synthesis. Based on previously published study from Dr. Kit Lam lab [9], we designed the following highly focused tetrapeptide library with N-terminal capped with MPUPA-OH for further improvement on binding affinity and specificity: MPUPA-$X_1X_2X_3X_4$. For each random site (X), we selected 10 amino acids, which made the total number of permutations of this library to be 10^4=10,000. Follow the synthesis method shown in **Fig.8a**, the corresponding encoded microdiscs array was successfully constructed and used for α4β1 integrin-binding peptides screening on Jurkat cells. The positive peptide sequence on microdiscs can bind to the α4β1 integrin on the membrane of cells which make the cells attached onto the surface of microdiscs. As shown in **Fig.8b**, after incubation of the chip with Jurkat cell suspension and removal of the unbound cells, some microdiscs were occupied by different amount of cells while others were blank. To find the peptide with stronger affinity, a competitive binding test was employed (**Fig. 8b**). The library was screened with Jurkat cells and then incubated with competing ligand Bio-1211 at various concentrations. Bio-1211 was a commercially available peptide ligand, with strong and specific binding affinity to α4β1 integrin. To identify the barcode patterns on microdiscs, the chip was incubated in guanidine HCl (8M) solution for several minutes to remove all attached cells and imaged again.

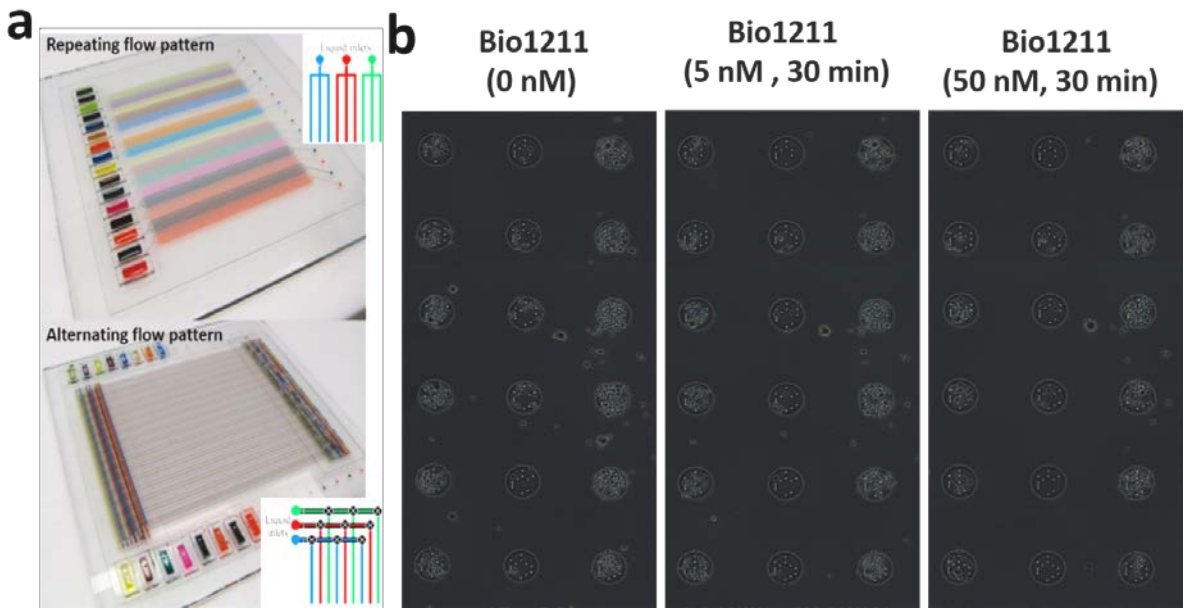

Figure 8. (a) Synthetic microfluidic patterns; (b) contrast images of microdiscs after cell screening.

In short, the success of peptide library array synthesis further confirms accessibility of ODOC for split-mix synthesis.

Key Research Accomplishments

1. Uniformly shaped microdisc carriers with biochemically activation and embedded barcode were fabricated and released in buffer solution to serve as peptide synthesis basis.

2. A microfluidic platform utilizing centrifugal force to split and pressurized liquid to mix microdisc carriers was built and proven to hold a volume of 16^4=65 536 individually unique carriers in four spinning motions.

Conclusion

Applying split-mix synthesis and barcode tracking, the synthetic history of each ODOC element can be digitally read out using scanning microscopy during every coupling step, which leads to exclusive barcode-to-chemical sequence mapping on corresponding microdiscs. This approach eliminates the need for identifying "positive-hit" molecules through chemical sequencing or mass spectrometry in the conventional OBOC, and at the same time may greatly increase the library size compared with array technique.

The microdiscs with inscribed barcodes can be batch-fabricated with extensive coding capacity by single-step photolithographic process of a PEG-based polymer, which is simple, reliable and low-cost. Utilizing the microfluidic split-mix synthesis and restricting chemical reactions onto microdisc surfaces, we can significantly facilitate the process of the peptide synthesis and reduce the reagents used. The structural barcodes also eliminate the need for interior chemical coding, thereby, requires less than one tenth of the quantity of building blocks needed for standard OBOC synthesis. Besides, a novel centrifugal microfluidic platform was introduced into the digital chemistry field and may make the split-mix peptide synthesis much easier and convenient. Peptide library array synthesis was also first tested which further confirmed accessibility of ODOC for split-mix synthesis.

In the future, we will first focus on developing the decoding algorithm for barcode recognition. A scanning system consisting of a microscopic setup and pattern recognition algorithm will be built. An Olympus IX81 inverted biological microscope equipped with PRIOR H117 motorized x-y stage will be employed to scan and image the barcoded discs during each synthetic step. At its travelling speed of 60 mm/s and exposure time of 4 ms, the motorized microscope (using a 4× objective) will be able to image an area of 400 mm^2 in 3 min, equivalent to capturing an array of 640 000 closely packed 100 μm circular microdiscs within an hour. The scanned microdisc images can be then processed and decoded using a pattern recognition algorithm programmed in Matlab. Second, a matured microfluidic synthesis platform should be developed for peptide synthesis and coordinated with barcode recognition system. The final goal is to combine large-scale combinatorial synthesis and digital molecular identification as a whole. Third, to prove the efficiency of the microfluidic split-mix peptide synthesis platform, we will then carry on a random cancer-targeting peptide

libraries synthesis, screening and optimization. A library of no more than 16^4=65 536 peptides can be synthesized during 4 individual split and mix period. Each individual microdisc carrier with a unique peptide sequence will be identified with decoding system. Also we will continue to improve our microfluidic device to accommodate even larger amount of microdiscs in one spinning motion.

Publications, Abstracts, and Presentations

(1) S. Zhao, Z. Bai, K. Lam, and T. Pan†, "Microfluidics-enabled Combinatorial Peptide Library for High Throughput Screening," Accepted, microTAS, 2014.

(2) S. Zhao, Z. Bai, K. Lam, and T. Pan†, "Digital One-Disc-One-Compound Array for High-Throughput Discovery of Cancer Cell Surface Targeting Ligands," 7th Annual Spotlight on Junior Investigator Cancer Research mini-Symposium, May 13, 2013, Davis, California.

Inventions, Patents and Licenses

N/A

Reportable Outcomes

N/A

Other Achievements

N/A

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Appendices n/a